

Anticoccidial and hepatoprotective effects of artemisinin liquid extract, cinnamon essential oil and clove essential oil against *Eimeria stiedae* infection in rabbits

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Abstract. Coccidiosis is one of the most dangerous diseases that affect poultry, resulting in worldwide economic losses. Plant extracts and essential oils have been used as potential alternatives for chemotherapeutics, because they don't have the negative consequence of creating tissue residue and drug resistance. Therefore, this study had been conducted to determine the efficacy of artemisinin liquid extract, cinnamon essential oil and clove essential oil against *Eimeria stiedae* in rabbits. Sixty New Zealand white rabbits were divided into six equal groups, where group 1 and group 2 represented the negative and the positive controls, respectively, and groups 3–6 were infected with *Eimeria stiedae* and received 15 ppm toltrazuril, 200 ppm artemisinin, 100 mg/kg cinnamon oil, and 100 mg/kg clove oil, respectively. The results showed that artemisinin had a significant beneficial role in protection against hepatic coccidiosis: it mitigated the clinical symptoms, reduced the mortality rates, improved body weight and feed conversion, decreased the oocyst output, prevented oxidative stress, improved biochemical parameters, and decreased the lesion formation. Moreover, it has been found that cinnamon and clove essential oils induced partial protection against hepatic coccidiosis. Our findings suggested that artemisinin liquid extract and cinnamon and clove essential oils could be used for protection against hepatic coccidiosis. However, further investigations are needed in order to elucidate the active components, optimal doses, and mode of action of these extracts and essential oils before their clinical applications.

INTRODUCTION

Poultry coccidiosis caused by the *Eimeria* species is among the most dangerous diseases worldwide and is responsible for high economic losses. For instance, in Egypt the disease caused around 11.83% mortality

rate and around 21.67% income losses (Fasina *et al.*, 2012). Moreover, the disease caused around \$127 million in annual losses in the United States' poultry industry and accounts for around \$2.4 billion in global economic losses annually (Abbas *et al.*, 2017; Ahad *et al.*, 2018). Coccidiosis is also

a major problem in rabbit farms, which seriously impairs rabbit's growth performance, feed utilization and cause high morbidity and mortality rates (Abdel-Megeed *et al.*, 2005). Unfortunately, coccidiosis in rabbits has not been studied extensively as compared to coccidiosis in other hosts (Al-Mahal, 2008). Coccidiosis in rabbits is associated with two anatomical forms: hepatic, which is caused by *Eimeria stiedae*, the most common species to infect rabbits and cause death (Al-Rukibat *et al.*, 2001), and intestinal, which is caused by other species like *E. intestinalis*, *E. irresidua*, *E. magna*, *E. perforans*, and *E. media* (Çam *et al.*, 2008).

Control of coccidiosis mostly depends upon chemoprophylaxis by the use of chemotherapeutic agents (Dalloul and Lillehoj, 2006); however, managerial skills are also reported to be of significant importance in achieving the maximum anticoccidial effects of these drugs (Tewari and Maharana, 2011). Although this chemotherapeutics were first used successfully, the development of resistance in the *Eimeria* species made these drugs less effective (Abbas *et al.*, 2017). In addition, most of the current anticoccidial drugs cause deleterious side effects (El Akabawy *et al.*, 2004) and are very expensive (Dalloul and Lillehoj, 2006). Moreover, drug residues in poultry products may be highly toxic to human beings. Therefore, there is growing interest in developing novel approaches to combat that problem and reduce economic losses. Several approaches have been developed, such as the use of natural products, live vaccines, probiotics, enhancing farm management practices, and improving the rabbit's immunity (Dalloul and Lillehoj, 2006). Recently, particular interest has been paid to antioxidant-rich botanical extracts as potent anticoccidial agents that show comparable results to synthetic drugs (Pérez-Fonseca *et al.*, 2016; Abbas *et al.*, 2017).

Among the potential candidates is artemisinin, which is a Chinese herbal extract that has been used for centuries in oriental medicine as an antipyretic and sedative agent and used for treatment of

human malaria, gastrointestinal helminthiasis, haemorrhoids, skin rashes, and diarrhoea (Galasso *et al.*, 2007; Zhang and Gerhard 2008; Arab *et al.*, 2009; del Cacho *et al.*, 2010). Although this plant extract demonstrated promising anticoccidial activities against avian coccidiosis (Arab *et al.*, 2006), little is known about its effect against hepatic coccidiosis. In addition, some essential oils (like clove and cinnamon) have different biological activities alongside their potent antioxidant effects, such as their antimicrobial, anti-cancer, and anti-inflammatory effects (Ranasinghe *et al.*, 2013; Cortés-Rojas *et al.*, 2014). Interestingly, these essential oils have never been tested against hepatic coccidiosis.

Here we report for the first time the effects of artemisinin liquid extract, cinnamon oil, and clove oil against *E. stiedae* infection in rabbits as compared to toltrazuril. Moreover, the growth performance, haematological, biochemical, and antioxidant effects of these extracts in rabbits were tested as well.

MATERIALS AND METHODS

Plant materials and commercial drug

Clove and cinnamon essential oils were purchased from (Haraz Company for Medicinal Plants, Cairo, Egypt). *Artemisia annua* (Artemisinin 20%®) watery extract was purchased from (Ibex Pharmaceuticals, Cairo, Egypt). Toltrazuril oral solution (Biocoxal 2.5%®): was purchased from (Universal Industries Pharmaceuticals Co., ElOubour City, Cairo, Egypt).

Isolation and preparation of *Eimeria stiedae* oocysts

Eimeria stiedae oocysts were collected from the liver nodules and the lumen of gallbladders of naturally infected rabbits. The infected rabbits were collected from commercial local rabbit farms which have history of *Eimeria* infections. The collected oocysts were washed and concentrated by the use of the flotation method (Heelan and Ingersoll, 2002). Sporulation of oocysts was

done in a moist chamber at 24–26°C for one week. The sporulated oocysts were stored in 2.5% potassium dichromate aqueous solution at 4°C until used to prevent fungal growth. The obtained sporulated oocysts were used to induce an experimental infection of rabbits. The oocysts count (oocysts per gram of faeces, OPG) was estimated by the use of the McMaster technique, according to Levine (1988).

Experimental animals

In this study, 60 healthy growing New Zealand White rabbits (*Oryctolagus cuniculus*) of both sexes (4-6 weeks old) with an average weight of 500–1000 gm and free from coccidian infection were used. The rabbits were obtained from the department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. To prevent direct contact with their faeces, the rabbits were housed in clean wire-floored batteries and were kept under the same nutritional, environmental, and hygienic conditions throughout the experimental period with controlled humidity (55-60°C), temperature (16-22°C), and light periods (12-h light/12-h dark cycles). The rabbits were fed pelleted commercial feed (Ibex Co., Cairo, Egypt) and water was supplied *ad libitum* throughout the experimental period. Faecal examination of rabbits by the use of concentration flotation technique was performed daily for 3 successive days prior to infection to prove that rabbits are *Eimeria* spp. free prior to the experiment. Animal rearing, handling, and all experimental designs were approved by the Research Ethical Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt.

Experimental design

The rabbits were weighed and randomly divided equally into 6 groups (n=10) as follows:

Group 1: Non-infected, non-treated (healthy control negative).

Group 2: Infected, non-treated (control positive).

Group 3: Infected and administrated toltrazuril liquid solution, at a daily dose of 15 ppm in drinking water, according to Peeters and Geeroms (1986).

Group 4: Infected and administrated artemisinin watery extract orally, at a daily dose of 200 ppm in drinking water.

Group 5: Infected and orally administered with clove essential oil, at a daily dose of 100 mg/kg which given orally by the use of a stomach tube to each rabbit daily.

Group 6: Infected and orally administered with cinnamon essential oil, at a dose of 100 mg/kg which given orally by the use of a stomach tube to each rabbit daily.

All groups (except group 1), were experimentally challenged with *E. stiedae* oocysts. The assessed plant's oils, extract and experimental drug were given to rabbits 5 days prior to challenge with *E. stiedae* and continued till the end of experiment. Challenge was performed by a single dose of 2.5×10^4 sporulated oocysts of *E. stiedae* per rabbit inoculated orally by a stomach tube on the sixth day after administration of the assessed plants and the reference drug. All groups were kept under observation for 29 days' post challenged (PC). Administration of drug and assessed oils and extracts were applied orally every morning after an overnight fast and before feeding.

Assessment criteria of preventive efficacy of the assessed plants and experimental drug:

1. Clinical signs

The signs of hepatic coccidiosis were recorded during the experimental period and beginning observed on the 15th day PC.

2. Mortality rate

The mortalities were recorded daily for each group and calculated as a percentage of mortality.

3. Growth performance

The rabbits were weighed at the beginning and the end of the experiment. Their body weight (BW), feed intake (FI), body weight gains (BWG) and feed conversion ratio (FCR) were recorded according to Davis *et al.* (1986).

4. Evaluation of oocyte reduction percentage

Three fresh faecal oocyst samples were collected from different parts of the litter spread on the battery tare daily from each group for oocysts count, from the day 17th post- challenge (PC), (When oocysts begin to be excreted in the faeces) until the day 29th pc (when only few or no oocysts could be detected in faeces). The collected faecal samples were stored in plastic containers labelled with group identification and date and transported to the laboratory for counting the mean number of oocysts per gram of faeces (OPG) for each group after being concentrated by the flotation technique and counted by the use of the McMaster counting technique according to the method described by Levine (1988). The reduction percentage of oocysts was calculated according to the number of oocysts per gram of faeces in control and treated groups.

Blood sampling

At the end of the experiment, after overnight fasting, two blood samples were collected separately from ear veins of randomly selected five rabbits of each group. One blood sample (1 ml) was collected in sterile tubes containing EDTA as anticoagulant for hematological analysis, the other (2 ml) were collected without anticoagulant for biochemical studies. After blood collection, the rabbits were sacrificed by decapitation. Livers were collected and rapidly excised from each animal, trimmed and divided into two parts; the first part was washed free of blood with 0.9% NaCl solution and distilled water to assess tissue oxidative status and antioxidant indices. The second part was used for histopathological examination.

Haematological assessment

The aliquot contained EDTA (1mg/ml) were used for assessing red blood cells (RBCs), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBCs), and blood platelets values were estimated by the use of the automatic cell counter (Orphee Mythic 22 CT Hematology Analyzer (Planles-Ouates, Switzerland) (Jain, 1986).

Biochemical analysis

The separated serum samples were used for colorimetric determination of liver enzyme markers by the use of commercially available kits (Merck and DiaSys Diagnostic Systems) according to the manufacturer's instructions. Serum aspartate amino transferase (AST), alanine amino transferase (ALT) were evaluated according to the method described by Reithman and Frankel, 1957, gamma glutamyl transferase (GGT) (Thomas, 1998), alkaline phosphatase (ALP) (Thirunavukkarasu *et al.*, 2003), serum Albumin level was quantified according to the procedure of (Chauhan and Chandra, 2007), and serum total protein content was measured by Lowry *et al.*, 1951.

Antioxidants and lipoperoxidation markers estimation

The dissected liver tissues were washed with 50 mmol/L sodium phosphate-buffered saline (100 mmol/L Na₂HPO₄/NaH₂PO₄, pH 7.4) in an ice-containing medium, with 0.1 mmol/L EDTA for removal of any RBCs and clots remnants. The tissues were homogenized in around 10 mL cold buffer per gram of tissue before centrifugation at 2000g for 30 min. The resulting supernatant were transferred into Eppendorf tubes, divided into aliquots and preserved at -80°C for assessment of oxidative stress markers. Antioxidants and lipoperoxidation markers were estimated calorimetrically by following the manufacturer's procedure (Biodiagnostics, Egypt). The activity of hepatic reduced glutathione (GSH), hepatic

superoxide dismutase (SOD), lipid peroxidation in liver homogenate (MDA) and catalase activity were estimated as described previously by Ellman (1959), Nishikimi *et al.* (1972), Ohkawa *et al.* (1979), and Sinha, (1987), respectively.

Liver index and lesion count

At the end of the experiment, five rabbits were weighed and sacrificed. The livers tissues were dissected, washed, inspected macroscopically and weighed. The liver index for each rabbit was estimated as wet liver weight divided by body weight in accordance to Gomez-Bautista *et al.* (1986). Specific nodules for hepatic coccidiosis were counted in the left lobe of liver of sacrificed rabbits. Impression smears which are collected from the cut surfaces of liver through the nodules were examined under high power objective of the microscope. The micrometric studies of representative samples and oocyst characteristics were conducted. Liver lesions were scored as previously described by Peeters and Geeroms (1986) and Mohammad *et al.* (2012).

Post mortem and histopathological examination

Tissue specimens from the livers of randomly picked up five rabbits from each group as well as all rabbits that died during the experiment were excised. The livers of the different groups were inspected for hepatic coccidiosis. The observed gross pathological changes were including abnormality in size, colour, consistency and typical nodules specific for hepatic coccidiosis. For hepatic histopathology, the tissue specimens were taken and processed for light microscopic examination. These specimens were fixed in 10% formalin before dehydration in ascending grading of ethyl alcohol, followed by clearing in xylene and embedded in paraffin. Finally, 4µm sections were obtained and stained with hematoxylin and eosin and trichrome stain to detect fibrosis (Dries, 2008). Histopathological changes were scored as none (-), mild (+), moderate (++), and severe (+++) damage.

Statistical analysis

All data were expressed as means ± S.E. and statistically analyzed by one-way ANOVA and Tukey multiple comparisons by the use of graph pad prism 5 software. Statistical significance was acceptable to a level of $P \leq 0.05$.

RESULTS

Clinical signs, growth performance, and mortality rate

In this study, no clinical abnormalities were observed in rabbits in the healthy control group (G1) and toltrazuril treated group (G3), where the animals remained healthy and showed normal appetite throughout the experimental period. However, rabbits in infected non treated group (G2) exhibited the typical symptoms of hepatic coccidiosis; depression, dullness, reduction of food intake, anorexia, wasting, emaciation, decreased activity, brown watery diarrhoea, ascites, polyuria, rough hair coat, hair loss, jaundice, pendulous with distended abdomen and bloating, accompanied with progressive weakness leading to death within 3-4 days. These symptoms were remarkably improved in artemisinin-supplemented group (G4) and moderately improved in cinnamon oil supplemented group (G5) and clove oil supplemented group (G6) (Data not shown). Moreover, there was a significant improvement of all growth performance rates including body weight, body weight gain, feed consumption and feed conversion in G4, G5 and G6 as compared with G2 (Table 1). The mortality rate was high in G2 (30%), which reduced to 10% in G4 and 20% in G5 and G6, while no mortalities were recorded in G3 (Table 1).

Oocysts count

The first oocyst output in faeces was observed on 17 day PC, and oocysts were shed in considerable amounts until the end of the study. There was a significantly ($P < 0.05$) higher oocyst output in G2 beginning from 17 day PC till the end of experiment compared to the other treated groups. Moreover, faecal oocyst count in G4,

Table 1. Body weight, body weight gain, feed consumption, feed conversion (mean \pm standard error) and mortality percent in control and treated groups

| Group | Body weight (g) | | Body weight gain (g/day) 0-35 day | Feed consumption (g) | Feed conversion (g) | Mortality (%) |
|-------|-----------------|------------------|-----------------------------------|----------------------|---------------------|---------------|
| | At 0 day | At 35 day | | | | |
| G1 | 952 \pm 67.6a | 1927 \pm 28.4a | 27.9 \pm 1.16a | 2982 \pm 0.3a | 3.05 \pm 0.003d | 0 % |
| G2 | 952 \pm 66.8a | 1443 \pm 30.8d | 14.0 \pm 1.15c | 2325 \pm 0.3f | 4.73 \pm 0.003a | 30% |
| G3 | 954 \pm 69.8a | 1920 \pm 21.9a | 27.6 \pm 1.40a | 2879 \pm 0.3b | 2.98 \pm 0.030e | 0% |
| G4 | 952 \pm 67.1a | 1800 \pm 20.5b | 24.2 \pm 1.37ab | 2532 \pm 0.3e | 2.98 \pm 0.026e | 10% |
| G5 | 956 \pm 65.1a | 1705 \pm 33.5c | 21.4 \pm 0.96b | 2546 \pm 0.3d | 3.39 \pm 0.026c | 20% |
| G6 | 953 \pm 78.9a | 1674 \pm 20.8c | 20.6 \pm 1.68b | 2609 \pm 0.3c | 3.61 \pm 0.026b | 20% |

Table 2. Oocysts count (mean \pm standard error) at one gram of faeces at different days in control and treated groups

| Group | Day | | | | | |
|-------|------------|------------------|------------------|------------------|------------------|------------------|
| | 0 | 17 | 20 | 23 | 26 | 29 |
| G1 | 0 \pm 0a | 0 \pm 0 e | 0 \pm 0 e | 0 \pm 0e | 0 \pm 0e | 0 \pm 0e |
| G2 | 0 \pm 0a | 34967 \pm 233a | 61633 \pm 441a | 70250 \pm 301a | 85267 \pm 318a | 80520 \pm 397a |
| G3 | 0 \pm 0a | 0 \pm 0e | 0 \pm 0e | 0 \pm 0e | 0 \pm 0e | 0 \pm 0e |
| G4 | 0 \pm 0a | 19173 \pm 525d | 19557 \pm 419c | 18467 \pm 536d | 17827 \pm 251d | 17510 \pm 296d |
| G5 | 0 \pm 0a | 26273 \pm 327c | 54617 \pm 553b | 58557 \pm 458c | 48100 \pm 503c | 34267 \pm 669c |
| G6 | 0 \pm 0a | 32700 \pm 321b | 55663 \pm 358b | 62167 \pm 273b | 49400 \pm 451b | 43477 \pm 930b |

Table 3. Liver weight, liver index and lesion count (mean \pm standard error) in control and treated groups

| Group | Body weight (g) | Liver weight (g) | Liver index | Lesion count |
|-------|------------------|------------------|------------------|-------------------|
| G1 | 1988 \pm 27.4a | 30.8 \pm 1e | 1.54 \pm 0.04e | 0 \pm 0c |
| G2 | 1369 \pm 24.4d | 112.0 \pm 6a | 8.20 \pm 0.47a | 43.2 \pm 13.04a |
| G3 | 1916 \pm 32.5a | 30.9 \pm 0.9e | 1.64 \pm 0.02e | 0 \pm 0c |
| G4 | 1824 \pm 23.7b | 45.6 \pm 0.7d | 2.50 \pm 0.07d | 19.0 \pm 6.75b |
| G5 | 1766 \pm 32.2b | 61.0 \pm 4.3c | 3.48 \pm 0.31c | 36.6 \pm 9.95a |
| G6 | 1627 \pm 18.1c | 79.0 \pm 2.9b | 4.84 \pm 0.20b | 34.8 \pm 6.48a |

G5 and G6 was significantly ($P < 0.05$) decreased when compared with those of G2, but the highest reduction was observed in G4 as shown in Table 2.

Liver weight, liver index and lesion count

Regarding to the effect of different treatments on liver weights and liver index, the results presented in Table (3) cleared that, G4, G5 and G6 were significantly decreased compared with G2. However, the lesion count was only significantly decreased in G4 as compared to G2.

Haematological parameters

With the exception of mild significant increase in RBCs count in G5, Hb concentration in G4 and non-significant increase in PCV in G4, G5 and G6, there was no change in haematological parameters in G4, G5, and G6 as was observed in G2 (Table 4).

Differential leucocytic count

The results in Table 5 showed a significant decrease in WBCs count G4 as compared to G2. Specifically, eosinophil count was

Table 4. Haematological parameters (mean ± standard error) in control and treated groups

| Groups | Parameters | | | | | |
|--------|-----------------------------|--------------|-------------|-------------|-------------|-----------------|
| | RBCs (x10 ⁶ /µl) | PCV (%) | Hb (g/dl) | MCV | MCHC | Blood platelets |
| G1 | 5.28±0.15ad | 33.6±0.52a | 10.9±0.25a | 63.82±1.07a | 32.4±0.31a | 223±44.59a |
| G2 | 4.22±0.10cb | 28.44±0.41b | 9.5±0.18b | 67.5±1.79a | 33.4±0.4a | 216±29.6a |
| G3 | 5.36±0.15a | 33.8±0.92a | 11.0±0.23a | 63.12±1.07a | 32.7±0.35a | 263±12.15a |
| G4 | 4.72±0.18ab | 31.18±0.84ab | 10.70±0.20a | 66.32±2.26a | 31.64±0.5a | 212.4±23.84a |
| G5 | 4.47±0.17a | 31.8±0.35ab | 10.6±0.15a | 71.58±2.8a | 33.27±0.31a | 330.6±16.88a |
| G6 | 4.65±0.14bd | 31.12±0.52ab | 10.88±0.16a | 67.12±2.26a | 34.98±0.57a | 308.8±29.8a |

Table 5. Differential leucocytic count (mean ± standard error) in control and treated groups

| Groups | Parameters | | | | |
|--------|-----------------------------|-----------------|-----------------|---------------|-----------------|
| | WBCs (x10 ³ /µl) | Eosinophils (%) | Neutrophils (%) | Monocytes (%) | Lymphocytes (%) |
| G1 | 8.12±1.2bc | 2.0±0.3d | 30.8±4.43a | 7.4±0.92a | 59.6±4.8a |
| G2 | 16.6±1.75a | 9.2±0.37a | 42.4±3.7a | 8.8±1.06a | 39.6±4.3a |
| G3 | 9.6±1.01c | 3.6±1.07dc | 41.8±2.69a | 7.2±0.49a | 46.8±2.5a |
| G4 | 9.8±0.77c | 5.2±0.25ce | 30.8±2.92a | 8.0±0.40a | 55.0±2.55a |
| G5 | 12.44±0.74abc | 6.4±0.5be | 41.0±4.12a | 9.2±0.37a | 44.0±4.26a |
| G6 | 12.4 ±0.72abc | 6.8±0.37abe | 33.6±2.71a | 7.2±0.73a | 50.0±2.34a |

significantly decreased in G4 and G5 as compared to G2.

Blood biochemical parameters

The recorded data in Figure 1 revealed a significant decrease in serum ALT, AST, ALP and GGT activities in G4 as compared to G2 at 29th day of infection. Total protein was significantly increased in G4 and G5 as compared to G2, while albumin showed non-significant changes in G2, G4, G5, and G6.

Liver antioxidant biomarkers

The results revealed that G4 had a significant decrease in the level of MDA, significant increase catalase activity and non-significant increase in SOD activity compared with G2. On the other hand, there were no significant differences in the antioxidant parameters in G5 and G6 compared with G2 (Figure 2).

Histopathology results

At necropsy, livers of G1 were of normal size with no obvious nodules or foci (Figure 3A). However, in rabbits infected with *E. stiedae* and non-treated (G2), livers were greatly

enlarged with presence of multiple variable sized nodules with distended gall bladders (Figure 3B), up on sectioning, these nodules were stuffed with yellowish green materials. While, in toltrazuril supplemented rabbits (G3), the livers were of normal size with no obvious nodules or surface changes (Figure 3C). In rabbits supplemented with artemisinin (G4), the livers were moderately enlarged with presence of fewer numbers of nodular lesions when compared with *E. stiedae* infected rabbits (G2) (Figure 3D). Moreover, in rabbits supplemented with clove (G5) or cinnamon essential oil (G6), there is no remarkable improvement in the livers size or the number of nodular lesions when compared with the livers of *E. stiedae* infected group (G2) (Figure 3E and F).

Histopathologically, the livers of control rabbits (G1) showed normally radiating hepatic cells around the central vein, with normal bile ducts and portal areas (Figure 4 A and B). However, in *E. stiedae* infected group (G2), there was generalized marked dilatation of bile ducts with papillary hyperplasia of their lining epithelium causing compression atrophy on the

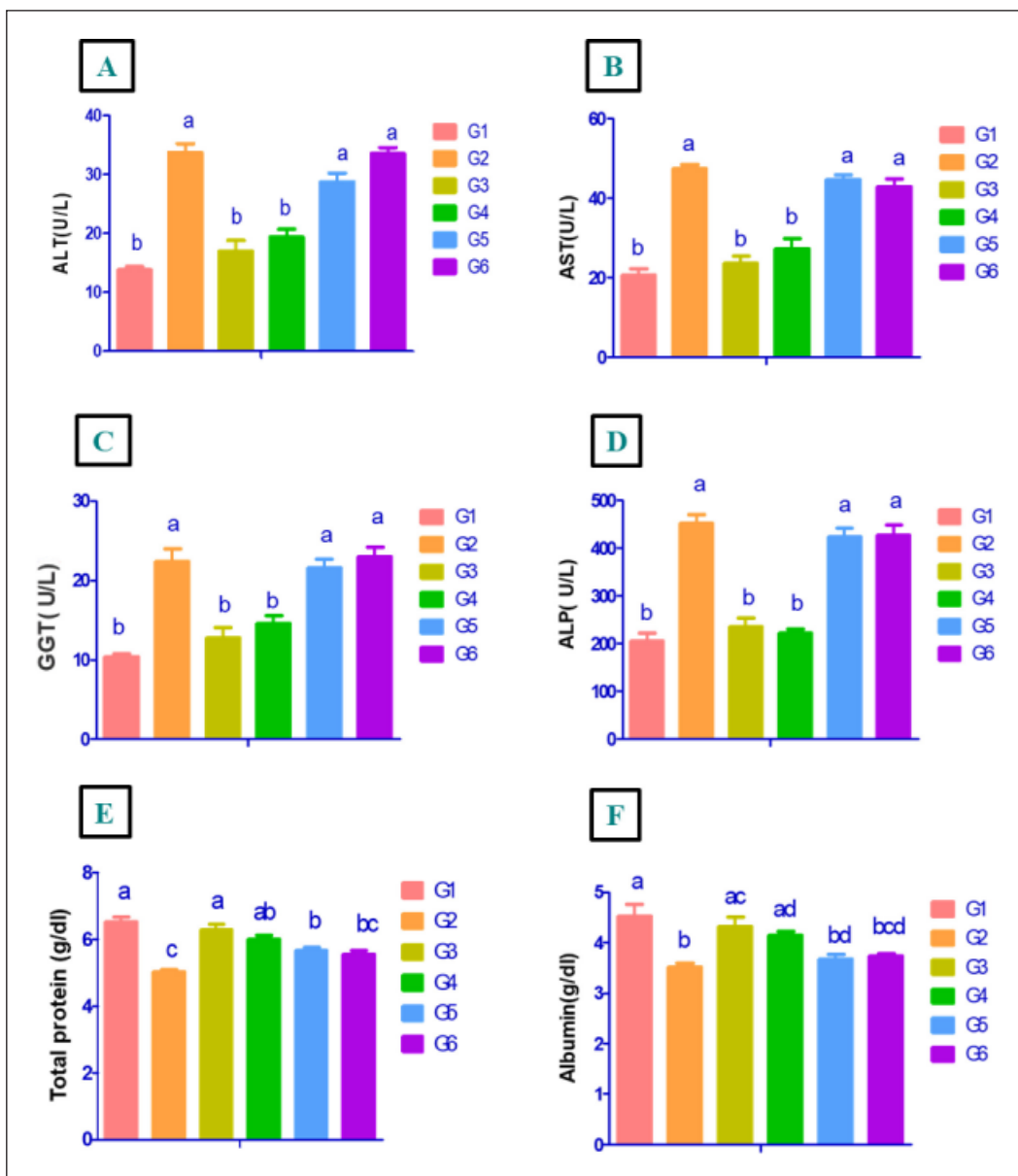


Figure 1. Blood biochemical parameters (mean \pm standard error) in control and different treated groups.

(A): Alanine amino transferase (ALT); (B) aspartate amino transferase (AST); (C) gamma glutamyl transferase (GGT); (D) alkaline phosphatase (ALP); (E) total protein and (F) albumin.

surrounding hepatic parenchyma (Figure 4C). The lining biliary epithelium and lumen were filled with different developmental stages of *E. stiedae* accompanied with massive periductal infiltrations of mononuclear cells most commonly lymphocytes (Figure 4D) and fibrosis (Figure 4E). The portal areas showed bile ducts proliferation

surrounded by massive periductal fibrosis and massive cellular infiltrations. The hepatic parenchyma adjacent to the over dilated hyperplastic bile ducts showed extension of cellular infiltrations with sinusoidal congestion and some vacuolar degeneration of hepatocytes in addition to focal areas of necrotizing hepatitis (Figure

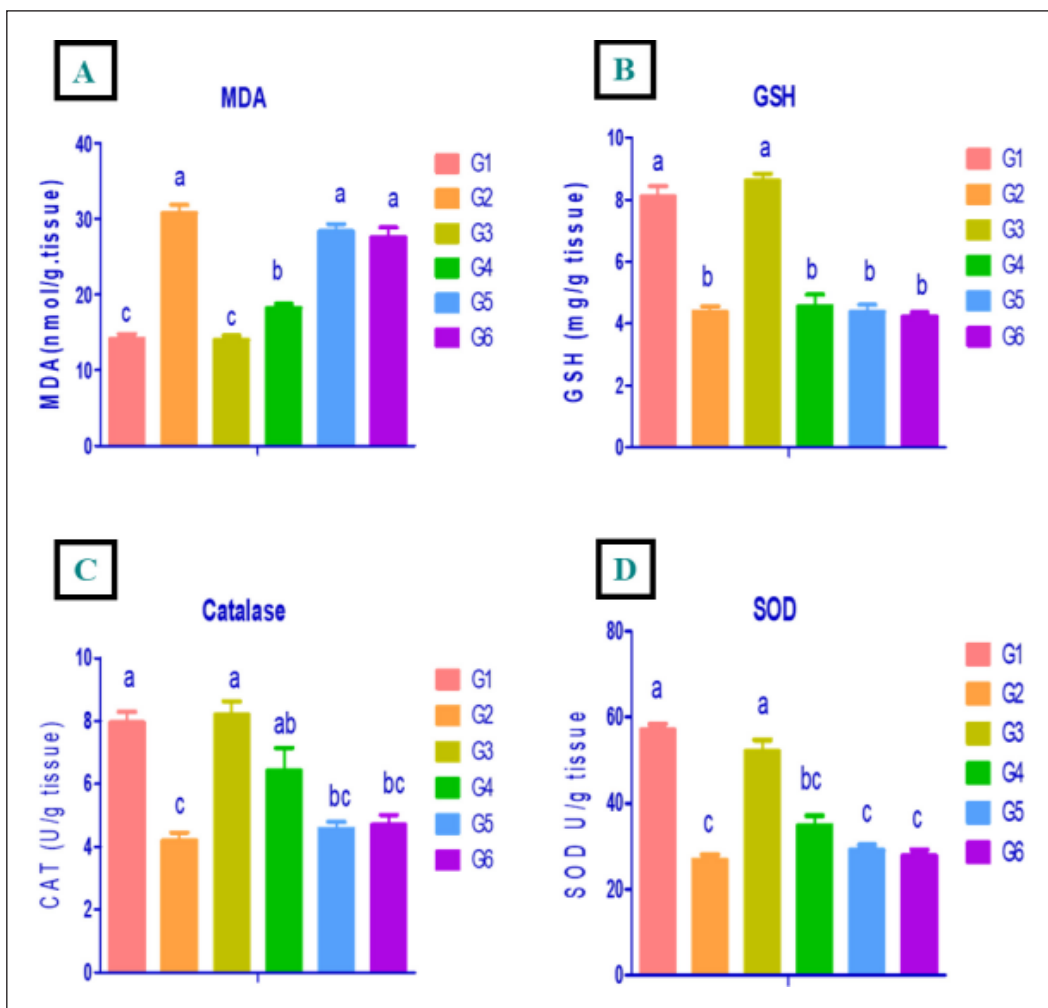


Figure 2. Hepatic antioxidant and lipid peroxidation markers (mean \pm standard error) in control and different treated groups. (A): Lipid peroxidation in liver homogenate (MDA); (B) hepatic reduced glutathione (GSH); (C) catalase and (D) hepatic superoxide dismutase (SOD).

4F). In toltrazuril supplemented rabbits (G3), there was no visible bile duct dilatation or any of developmental stages of coccidia except of minimal cellular infiltrations around bile ducts. However, in artemisinin-supplemented group (G3), there were decrease in the number of moderately dilated bile ducts, hyperplasia of their lining epithelium and amount of proliferating fibrous tissue (Figure 5A) but marked periductal cellular infiltrations were observed (Figure 5B). In both clove (G5) and cinnamon (G6) oil supplemented groups, wide spread bile duct dilatation,

multiple focal portal bile proliferation with cellular infiltrations and vacuolar degeneration specially in clove oil supplemented group (Figure 5C and D) and fibrous tissue proliferation were still visible but of less degree in cinnamon treated group, these changes are summarized in Table (6).

DISCUSSION

Coccidiosis is still a serious problem and encountered for high deaths in rabbit farms in many countries in the world. Previous

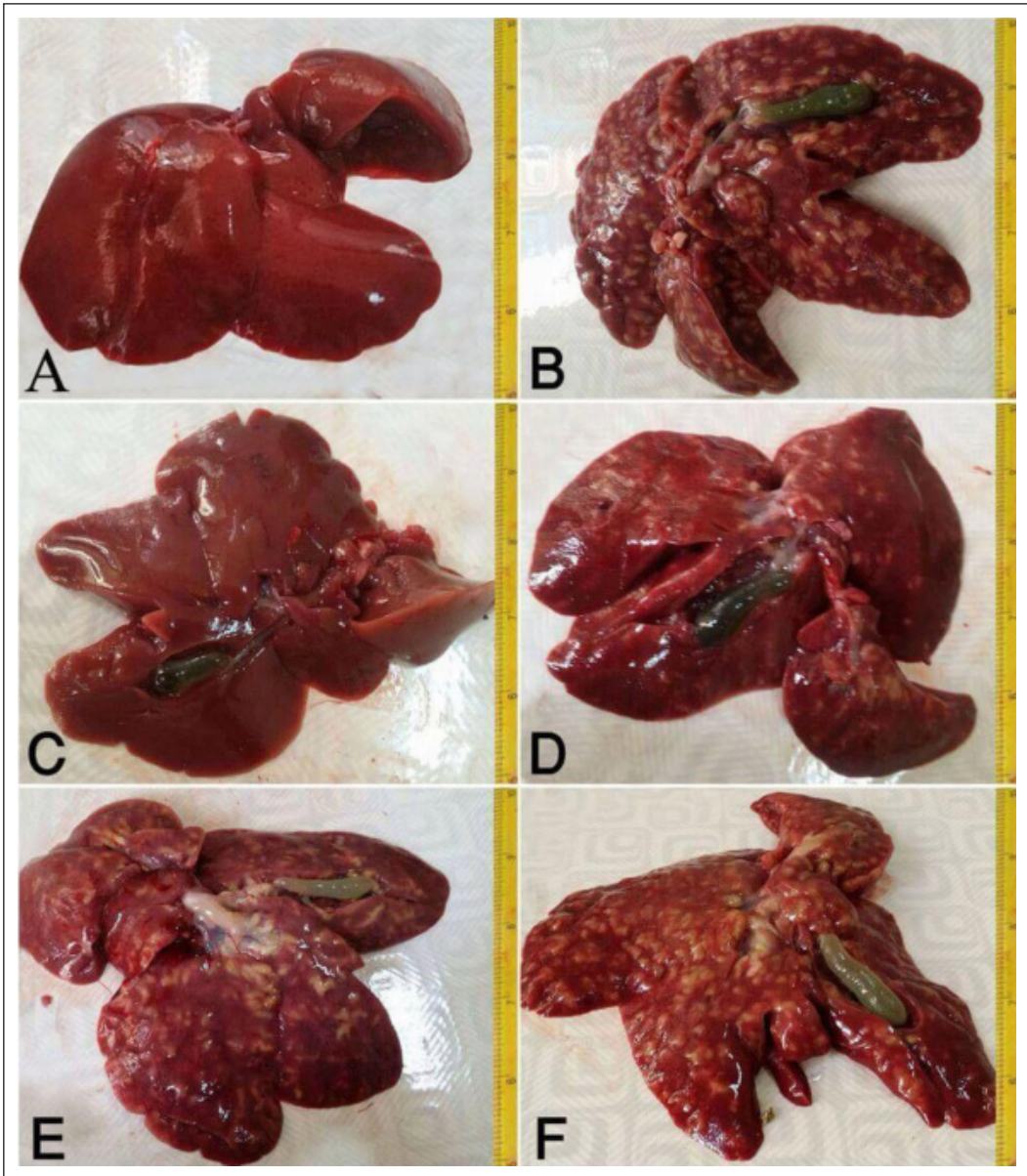


Figure 3: Gross pathology of the livers in different experimental treatments in *E. stiedae* infected rabbits.

(A): Control group, liver showing normal size with no observed lesion.

(B): *E. stiedae* infected group, liver showing marked enlargement with distended gall bladder and presence of multiple variable sized nodules.

(C): Toltrazuril treated group, liver showing normal size with no observed lesion.

(D): Artemisinin supplemented group, liver showing moderately enlargement with presence of fewer numbers of nodular lesions.

(E and F): Clove oil and cinnamon supplemented groups, liver showing marked enlargement with presence of nodular lesions.

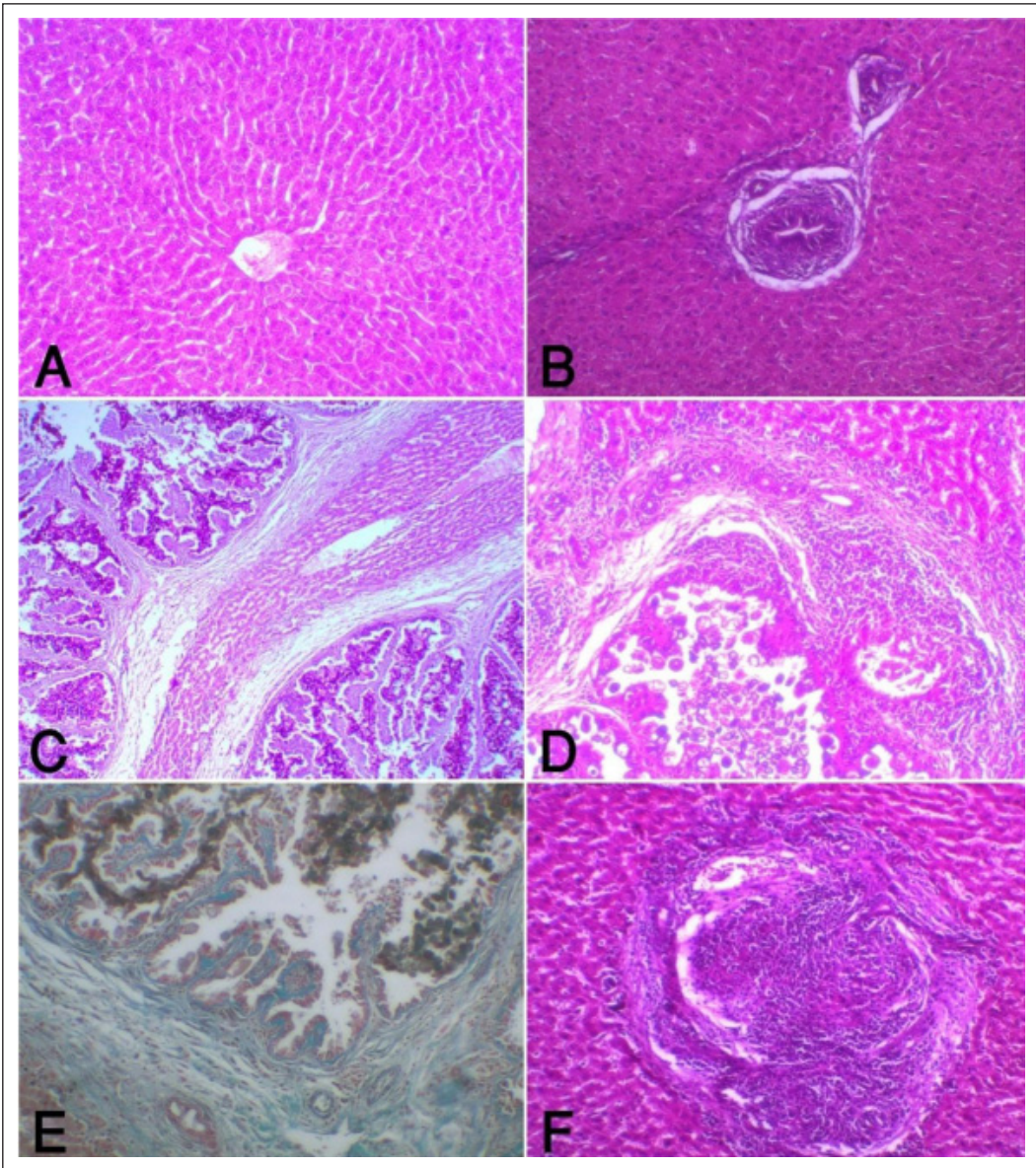


Figure 4. Histopathology of the liver in different experimental treatments in *E. stiedae* infected rabbits.

(A): Control group, liver showing normal radiating hepatic cells around the central vein, HE, x10. (B): Control group, liver showing normal bile ducts, HE, 10x10. (C): *E. stiedae* infected group, liver showing dilated bile ducts with hyperplasia of their lining epithelium containing different development stages of coccidia and compressed hepatic parenchyma, HE, 10x4. (D): *E. stiedae* infected group, liver showing the lining biliary epithelium and lumen were filled with different developmental stages of *E. stiedae* accompanied with massive periductal infiltrations of mononuclear cells most commonly lymphocytes and fibrosis, HE, 10x10. (E): *E. stiedae* infected group, liver showing massive periductal fibrosis, Trichrome stain, 10x20. (F): *E. stiedae* infected group, liver showing focal hepatic necrosis accompanied with massive mononuclear cells infiltration, HE, 10x10.

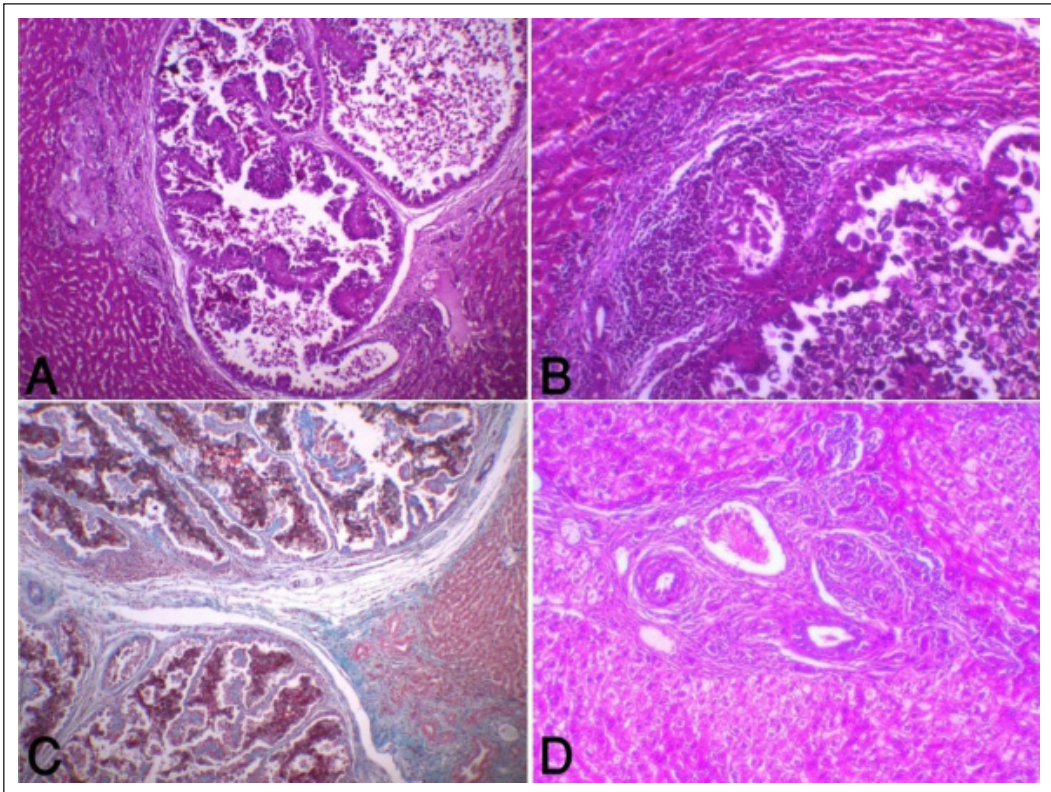


Figure 5. Histopathology of the liver in different experimental treatments in in *E. stiedae* infected rabbits.

(A): Artemisinin supplemented group, liver showing moderately dilated bile ducts and hyperplasia of their lining epithelium with minimal proliferating fibrous tissue, HE, 10x4. (B): Artemisinin supplemented group, liver showing marked periductal cellular infiltration, HE, 10 x10. (C): Clove oil/ cinnamon supplemented groups, liver showing markedly dilated bile ducts with hyperplastic epithelium containing coccidial developmental stages, HE, 10x4. (D): Clove oil/ cinnamon supplemented groups, liver showing proliferation of bile ducts in portal areas surrounded by massive cellular reaction and the adjacent hepatic parenchyma showed vacuolar degeneration specially in clove oil supplemented group, HE, x10.

Table 6. Summary of histopathological findings in different experimental groups

| Pathological findings | Groups* | | | | | |
|------------------------------------|---------|-----|----|-----|-----|-----|
| | G1 | G2 | G3 | G4 | G5 | G6 |
| Bile duct dilatation/proliferation | - | +++ | - | ++ | +++ | +++ |
| Biliary hyperplasia | - | +++ | - | ++ | +++ | ++ |
| Cellular infiltrations | - | ++ | + | +++ | +++ | ++ |
| Fibrous tissue proliferation | - | +++ | - | ++ | +++ | ++ |

* Scoring was done as follows: (-) none, (+) very mild, (++) moderate, (+++) severe.

studies have shown that antioxidant rich plant extracts and essential oils have promising anticoccidial potential activities and can be used as best substitutes to synthetic anticoccidial drugs (Abbas *et al.*,

2017). In addition, these extracts are natural, save, more appetizing with no residual effect comparing with synthetic chemical drugs (Pérez-Fonseca *et al.*, 2016; Abbas *et al.*, 2017). Therefore, the current study was

designed to demonstrate the anticoccidial effects of different plant extracts and essential oils including artemisinin liquid extract, cinnamon oil and clove oil against experimentally induced hepatic coccidiosis in rabbits.

The results of the existing study indicated that the progression of *E. stiedae* induced infection might be determined by alterations in the clinical, hematological, biochemical, lipid peroxidation and histopathological findings. In the current study, artemisinin extract has a potent anticoccidial effect and improved both of weight gain and feed conversion. Moreover, slight clinical manifestation of illness all over the experimental period, decreased lesion count, and oocyst output which are in bargains with Allen *et al.* (1997) and Abousekken *et al.* (2015). The current observations suggested that artemisinin extract and toltrazuril blocked the production of coccidian sexual stages in the liver which in turn reduced the number of oocysts in faeces. Also, it may be attributed to the potent antioxidants content of artemisinin that could limit the growth and development of *E. stiedae* stages leading to reduction of oocysts formation and their appearance in the faeces. Artemisinin may induce a state of oxidative stress via the extremely reactive free oxygen radicals cascade produced by the reductive cleavage of its highly reactive endoperoxide bridges by iron complexes or iron producing free radicals (Levander *et al.*, 1989), and to the capability of the free radical to alkylate protein causing death of the parasite (Galasso *et al.*, 2007; Zhang and Gerhard 2008; del Cacho *et al.*, 2010). On the other hand, cinnamon and clove oils supplementation had mild protective effects against *E. stiedae*, however, rabbits that received these oils showed improvement in liver weight, liver index and the performance which may be attributed to the higher percentage of fatty materials in the cinnamon and clove oils. Numerous prior studies testified the significant use of cinnamon and clove oils or their main constituents on the performance of broilers and their potential use as feed additives

(Lee *et al.*, 2004; Al-Kassie and Jameel, 2009). To our knowledge this is the first study report on studying the protective effects of Cinnamon and clove oil against *E. stiedae*.

Infection with *E. stiedae* induced significant decrease in haematocrit, haemoglobin and RBCs values, insignificant changes in the mean corpuscular volume, mean corpuscular haemoglobin concentration. Erythrocytes indices revealed a normocytic normochromic anaemia, which is a common complication in patients with chronic disorders as inflammation, infection, or various systemic diseases such as chronic liver disease. Multiple mechanisms contribute to anaemia associated with inflammation. The principal pathogenesis is believed to be related to hepcidin molecule that consider, a key molecule in controlling iron absorption and recycling. It released by the liver in response to inflammation and induced by the pro inflammatory cytokines. Alterations in proinflammatory cytokines production such as; interleukins (IL-1), (IL-6); tumour necrosis factor alpha (TNF); interferons, decreased the responsiveness of bone marrow to erythropoietin, and impaired iron availability to the erythron are all involved in the pathogenesis of anaemia. (Adamson, 2008; Bergamaschi *et al.*, 2008). On the other side, the total leucocyte counts and eosinophil percentage were significantly increased in hepatic coccidiosis. This was attributed to the either severe hepatic tissue damage that stimulate a variety of cells to release interleukins, growth factors, cytokines, and other mediators of inflammation that are interrelated in causing neutrophil proliferation, maturation, and release from the bone marrow into blood. These cells subsequently emigrate from the blood into tissues or endogenous release of cortisol during inflammation. Moreover, haematological parameters were markedly improved after toltrazuril supplementation and moderately improved after artemisinin supplementation. Similar improvement in haematological parameters was also recorded after toltrazuril treatment (Çam *et al.*, 2008) or other natural extracts such

as oleo-gum-resin from *Commiphora molmol* (Al-Mathal, 2010). Moreover, the noted data in the current study revealed lowered serum enzyme values of (ALT, AST, ALP and GGT) in toltrazuril and artemisinin treated groups as compared to infected non treated group (Figure 1). The improvement of blood serum enzymes can be attributed to the recovery of hepatic cells and epithelial lining and inhibition of the parasite sexual stages development. The recovery included healing of damaged liver tissues, bile ducts (the main site of infection), and normalization of many biochemical parameters levels, including liver enzymes and blood serum proteins following treatment. These results are similar to Çam *et al.* (2008) and Abdel-Maged *et al.* (2013). In addition, Faris *et al.*, 2011 documented that coccidiosis in kids induced a significant increase in serum AST, ALT, alkaline phosphatase, urea and creatinine. The current observations revealed that the tested all biochemical parameters returned to their normal levels after treatment by toltrazuril which approve its anticoccidial efficacy and hepatoprotective effects.

Oxidative stress is an important contributory factor to the pathophysiology of a variety of pathological conditions and defined as an increase in reactive oxygen species (ROS) and/or a decrease in the anti-oxidant defence mechanisms (Heyman *et al.*, 2011). In this study, administration of sporulated oocyst of *E. stiedae* to normal rabbits (G2) exhibited a significant increase in liver MDA concentration, however liver catalase, GSH and SOD activities showed significant decrease after 29 days of infection compared to control group (G1) (Figure 2). These observations were hand by hand to that of Çam *et al.* (2008) and Abdel-Maged *et al.* (2013) who reported that, the increase in plasma MDA levels in the infected rabbits suggested that *E. stiedae* caused lipid peroxidation resulting from the destruction of the bile duct and subsequently the hepatic parenchyma. The peroxidation of cell membrane causes impairment in its permeability and triggers a series of reactions that may result in cell death. In addition, the decrease in GSH, CAT and

SOD activities at the end of experiment in this study might be associated with excessive free radicals releasing during the infection or a decrease in production of these enzyme as a result of liver damage (Kaya *et al.*, 2007; Wang *et al.*, 2008). Regarding toltrazuril supplemented group, the results of lipid peroxidation parameters were close to control values. However, artemisinin induced only decrease in the levels of MDA and increase in catalase levels which may also confirm its anti-oxidant and anticoccidial effects.

In the existing study, the livers of rabbit infected with *E. stiedae* were severely enlarged with distended gall bladders and the hepatic surface was covered with multiple varying sized nodules on gross findings when compared with control livers, which is in line with (Kardena *et al.*, 2015). Hepatomegaly is a typical lesion of *E. stiedae* infection due to extreme biliary hyperplasia and fibrosis (Al-Naimi *et al.*, 2012; Kardena *et al.*, 2015). Microscopically, the livers had severely dilated bile ducts with papillary hyperplasia of their lining epithelium where the ductal lumen and epithelium were filled with different developmental stages of coccidia which may be due to the predilection site of proliferation of the *E. stiedae* organisms is within the epithelium. The hepatic parenchyma in between the severely dilated ducts were atrophied, other parts of parenchyma showed congestion, focal necrotic hepatitis accompanied with mononuclear cells infiltration, along with sinusoidal cells activation with marked peri-biliary chronic inflammatory reaction which is characterized by widespread fibrosis and mononuclear cell infiltrations which are in agreement with (Al-Mahal, 2008; Al-Naimi *et al.*, 2012). These lesions may be due to liberated toxins or mechanical irritation aroused by coccidia (Sastri, 1983). Also, these lesions may be due to lipid peroxidation caused by *E. stiedae* which results in the destruction of the bile ducts and consequently the hepatic parenchyma and these lesions were completely ameliorated by toltrazuril treatment, where the liver showed no visible lesions related to hepatic coccidiosis although a few

oocysts were detected in the bile duct epithelial cells which lead to impairment in its permeability and triggers a series of reactions that may result in cell death (Çam *et al.*, 2008). However, moderate improvement by artemisinin supplementation were observed, where the size and number of dilated bile ducts, biliary hyperplasia and periductal fibrosis were reduced, however, extensive cellular reaction was remarkably seen. The noted hyperplasia may be recovered by time as suggested by Seddiek and Metwally (2013).

Artemisinin, acts as anti-coccidial agent through degradation of iron-implicated peroxide complex leading to interfering with the life cycle of *Eimeria* through inhibition of oocyst wall formation and sporulation (Allen *et al.*, 1997; del Cacho *et al.*, 2010). Also, *A. annua* has lots of phytochemicals, flavonoids, and phenolic compounds which can help birds to maintain commensal microflora and take up large amounts of nitrogen which in turn enhancing digestion of food and absorption of nutrients and improving innate and acquired immune response in poultry (Brisbin *et al.*, 2008). Cinnamon and clove oil supplementation have a very mild protective effect against *E. stiedae*. However, cinnamon oil showed reduction in the degree of biliary hyperplasia, fibrosis and cellular reaction, while, the wide spread dilated ducts were observed. Two phytonutrient mixtures of (carvacrol, cinnamaldehyde, and capsicum oleoresin) and (Capsicum oleoresin and turmeric oleoresin) treatments, were effectively protected against *E. tenella* infection by increase in NK cells, macrophages, CD4+ T cells, CD8+ T cells, and their cytokines (IFN- γ and IL-6) and a decrease in TNFSF15 and IL-17F, leading to induction and elevation of host immunity to kill *E. tenella* in chickens (Lee *et al.*, 2011). From these observed findings, we can conclude that oral administration of toltrazuril completely protects against hepatic coccidiosis in rabbits followed by artemisinin which partially clear the infection. However, clove oil and cinnamon weren't effective as a treatment. To our

knowledge, there are no previous reports studied the protective effect of cinnamon or clove oil supplementation against induced coccidia infection in rabbits.

CONCLUSION

In the current study, the infected group treated with toltrazuril showed no abnormal clinical findings and reduced the number of OPG to zero. In addition, hematological, biochemical, antioxidants and lipid peroxidation parameters of this group approximated to those of the healthy group. This positive effect was also supported by histopathological and macroscopic findings since there were no significant lesions in the liver parenchyma and bile ducts, suggesting that this treatment was effective. Thus, it is concluded that toltrazuril used at preventive dose of 15ppm in drinking water was very effective for the prevention of hepatic coccidiosis. Moreover, administration of artemisinin extract to infected rabbits caused a partial protection, as it alleviated the clinical symptoms, decreased the mortality, improved cumulative body weight, body weight gains and feed conversion, decreased the oocyst output, prevented oxidative stress and improved biochemical parameters. In addition, it decreased the lesion formation. However, cinnamon and clove oils had a mild effect as compared with toltrazuril reference drug and caused slight protection against hepatic coccidiosis. Therefore, further studies are necessary to assess the possible anticoccidial action of the cinnamon and clove essential oils, their active materials as well as optimal doses and mode of action and appropriate timing for employing the treatment and their value as an alternative or adjunct in therapeutic or prophylactic strategies.

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Conflict of interest statement

The authors report no conflicts of interest associated with this manuscript.

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